Protein Turnover in Grass Leaves

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ABSTRACT

In this chapter, we discuss the processes of protein synthesis and degradation at the cellular, organ and whole-plant levels. In particular, we focus on the leaf protein Rubisco, which is important as both the most abundant form of N in most leaves and the carboxylating enzyme in photosynthesis. Chloroplasts contain the largest fraction of cellular N, divided approximately equally between soluble protein and thylakoid-associated N. Recently, small vesicles have been noted emanating from chloroplasts; however, there is considerable debate on the properties and regulation of these bodies. Similarly, recent investigations into the turnover of the D1 protein have questioned the orthodoxy view that D1 turnover is caused by oxidative fragmentation. The final two sections of this chapter look into the factors influencing the patterns of protein synthesis and degradation at the whole-leaf and whole-plant levels, and the implications that has for plant growth, development and productivity.

I. INTRODUCTION

In most environments, nitrogen (N) is the predominant element limiting plant growth and, in agronomic systems, yield (Addiscott et al., 1991). The majority of N in most plants is stored in aboveground organs, primary among them are leaves. In cereals, the aboveground biomass may contain as much as 80% of plant N, with leaves accounting for approximately 60% of that. Leaf N concentration scales with photosynthetic capacity (Wright et al., 2004), and the N content of plants is closely related to their nutritional quality for animals, including humans.

N remobilization is important as an N supply for new growth, with over 60% of new leaf N being derived from protein turnover in older leaves (Mae, 1986). A recent paper showed that the average period in which N had been in the plant prior to its incorporation into a new leaf was 14 days, compared to 3 days for C (Lattanzi et al., 2005). Similarly, remobilized old leaf N has also been shown to be the main N source for new spring growth in perennial grasses (Bausenwein et al., 2001). For these reasons, in this chapter we shall discuss the processes of N remobilization in plant leaves. We shall focus on grass plants although we will use other species where they provide a useful example. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC4.1.1.39) will be especially focused on for two reasons: (i) Rubisco is the carboxylating enzyme in photosynthesis; and (ii) due to its low enzymatic rate, Rubisco is the most abundant enzyme not only in most plant tissues but in the world (Ellis, 1979). A third factor making Rubisco interesting is its method of production, with its small subunits encoded in the nuclear genome and translated in the cytosol, while large subunit biosynthesis takes place in the chloroplast.

This chapter is split into the following main sections: Section II discusses the regulation of protein synthesis at the cellular level, while Section III does the same for degradation. Sections IV and V will look at the integration of synthesis and degradation at the whole-leaf level and the implications of differential N turnover at the whole-plant level, respectively.

At this point, I would like to note that this chapter is not intended to be comprehensive in its treatment of this subject. The processes described in this chapter are becoming better and better investigated every day, and the volume of information available on this subject is simply too large for even a single book, let alone one review paper. This chapter would be better thought of as an introduction to the subject, providing readers with the opportunity to branch out and explore individual subjects at will.

II. PROTEIN SYNTHESIS

A. CELLULAR REGULATION OF PROTEIN SYNTHESIS

Within the leaf, a vast majority of N, perhaps 70% or more, is found within the chloroplast. Of that, the majority, perhaps three-quarters, is associated with photosynthesis (Makino and Osmond, 1991). Endosymbiotic theory tells us that all eukaryotic cells result from ancestral bacteria taking up residence inside other cells, probably around a billion years ago. This accounts for the double-membrane structure of higher plant chloroplasts, with the inner membrane being derived from the ancestral bacteria and the outer membrane deriving from the host cell (some marine microalgae have a four-membrane structure, revealing that the original endosymbiont underwent a secondary endosymbiosis). In accordance with their bacterial ancestry, eukaryotic organelles, specifically mitochondria and chloroplasts, maintain their own genomes. These genomes are much reduced compared to extant bacteria, as gene functions, if not genes themselves, were transferred from the bacterial genome to the nuclear genome over evolutionary time. The chloroplast genome encodes approximately 100 chloroplast proteins, while nuclear genes encode a number probably 30 times higher (Aluru et al., 2006). The expression of the nuclear and chloroplast genomes must be synchronized in their action during the cells lifetime, although it is not well understood how this is regulated. However, communication between the chloroplast and the nucleus is known to be bidirectional. Bacteria use a class of proteins known as sigma factors to regulate the activity of RNA polymerase, and these sigma factors have also been found in the higher plant nuclear DNA (Lysenko, 2007). Free sigma

factors 1–3 do not bind to the plastid DNA, but rather they bind with RNA polymerase, which causes a conformational change allowing them to bind later with the plastid DNA. The mechanism of action of the sigma factors 4–6 remains unclear. *Sig6* mutants are known to have decreased *rbcL* and *psbA* transcript abundances, suggesting that this gene may be important in nucleus to chloroplast signalling in developing leaves. The regulation of Rubisco synthesis, further discussed below, is a good example of chloroplast behaviour being modulated by the behaviour of the nucleus, while the downregulation of the nuclear light-harvesting complex b gene (*Lhcb*) and the Rubisco small subunit (RBCS) gene (*RbcS*) in response to tissue treatment with a carotenoid biosynthesis inhibitor (La Rocca et al., 2004), and in chloroplast defective mutants, demonstrates chloroplast to nucleus signalling (Aluru et al., 2006). Carotenoid biosynthesis mutants, such as the *immutans* mutant line of *Arabidopsis*, suffer photo-oxidative stress, leading to chloroplast bleaching, with sections of *immutans* leaves losing all their green chlorophyll colouration. We might also expect that nuclear genes which are responsive to light follow some kind of chloroplast to nuclear signalling.

In grasses, new cells are produced during cell division, which takes place at the base of the leaf. New cells contain about 15 small spherical pro-chloroplasts, 1–2 µm in diameter. Mature cells contain up to 60 chloroplasts per cell, with a size range of approximately 6–8 µm. During this maturation process, chloroplast DNA increases rapidly, up to 7.5-fold per chloroplast in wheat, which is presumably important in the rapid production of the vast quantities of protein which must be synthesized for photosynthesis. In illuminated barley leaves, the number of plastid DNA copies was maximal in cells at the base of the leaf and decreased as the cells matured. However, in leaves expanding in darkness, this decrease in plastid DNA copy number with cell age was not noted (Shaver et al., 2008). After this initial increase, the genome copy number rapidly decreases upon exposure to blue or white, but not red light (Oldenburg et al., 2006). In dividing cells, the rate of plastid transcription is very low; however, as the chloroplasts start to mature, transcription activity increases approximately 10-fold per plastid, and due to the increased number of plastids, approximately 30-fold per cell. This upregulation in transcription precedes increases in RNA levels, ribosome numbers and photosynthetic proteins (Baumgartner et al., 1989), and appears to be light-regulated (Dubell and Mullet, 1995). Sigma factors would seem to be implicated in this light signalling, with some sigma factors considerably upregulated upon illumination (Lysenko, 2007). Additionally, many sigma factors are only expressed in illuminated tissues, such as leaves, but not in roots.

Higher plant Rubisco is composed of eight large (Rubisco large subunit (RBCL)) and eight small subunits (RBCS). The large subunits are encoded by the chloroplast genome (plastome), whereas the small are encoded by the nuclear genome. The small subunit RNA is translated into protein in the cytosol and then imported into the chloroplast through the chloroplast membrane, losing its transit peptide in the process (Highfield and Ellis, 1978). *rbcL* mRNA is translated in the chloroplast, by bacterial type 70S ribosomes. RBCS protein entering the chloroplast binds with the RBCL, which is otherwise quickly degraded in the absence of RBCS. RBCL excess represses the translation of further *rbcL*, which seems generally to be in excess (Wostrikoff and Stern, 2007). While this system in which unassembled proteins are unstable and are rapidly degraded is common in many organisms, unassembled cytochrome *f* protein is no less stable than the assembled protein; however, its transcription is massively decreased in the absence of its assembly partners (Choquet and Vallon, 2000; Choquet et al., 2003). This downregulation of protein synthesis is known as "control by epistasy of synthesis" (CES). CES appears to be very important in the control of thylakoid membrane protein synthesis.

Many historical studies have used antisense mutant plants to investigate the subunit control of Rubisco biosynthesis (Hudson et al., 1992; Makino et al., 1997b; Quick et al., 1992) and its downstream effects on photosynthesis; however, only recently have the opposite *rbcS*-overexpressing plants been successfully produced. Suzuki et al. (2007), using *rbcS*-overexpressing plants, noted a 2.1–2.8-fold increase in *rbcS*, a 1.2–1.9-fold increase in *rbcL* and a 1.3-fold increase in Rubisco content. This suggests that Rubisco synthesis is regulated at several levels. *rbcL* levels were upregulated by *rbcS* overexpression; it seems likely that excess RBCS bound with RBCL which would be otherwise in excess, thus increasing the Rubisco protein levels and derepressing the production of *rbcL*. Rubisco concentrations increased by only 70% of the increase in *rbcL* transcript, however, suggesting a further level of regulation, which has been suggested to be mainly controlled by leaf N-influx (Imai et al., 2005). Of course, the rate of N import into a leaf represents an upper limit to the total amount of N-containing molecules which can be produced by the leaf, and so perhaps this result is unsurprising. Tobacco *rbcS* mutants with only 5% of wild-type *rbcS* transcript exhibited wild-type levels of *rbcL* transcript, but only 5% of wild-type RBCL protein (Wostrikoff and Stern, 2007). Similar results have also been noted in other studies. For example, *rbcS* antisense plants produce significantly less *rbcS* mRNA than control plants, but the levels of *rbcL* transcript were unchanged (Rodermel et al., 1996). *RbcS* also utilizes post-transcriptional control to regulate protein levels, with *Chlamydomonas* cells

unable to produce the large subunit due to a lack of 70S ribosomes exhibiting decreased *rbcS* mRNA levels (Mishkind and Schmidt, 1983).

Rubisco *rbc* gene expression exhibits a strong diurnal fluctuation (Cheng et al., 1998). In *Arabidopsis, rbc* levels increase overnight, then decrease sharply upon illumination—especially those of *rbcS* 1B. Following this initial decline, *rbc* transcript abundances increase in an almost linear manner in *rbcS* 1A and 1B. Although several control mechanisms can be postulated for these patterns of transcript abundance, this linear phase suggests that transcription may take place in a largely constitutive manner. However, the regulation of *rbcS* 2B and 3B appears to be more complicated, although the reason for this remains unclear. Both the fluence rate and wavelength are important, with blue light shown to be important in the upregulation of *rbcS* transcript abundance, whereas red and white light were less important (Lopez-Juez et al., 2007; Sawbridge et al., 1994). Various classes of photoreceptors are known to be active in plants, and these have been implicated in the regulation of both gene transcription (Spalding and Folta, 2005) and proteolysis (Huq, 2006). Grass leaves tend to green up and produce Rubisco as they exit from the previous leaf sheath (Gastal and Nelson, 1994) and it seems plausible that the mechanism promoting Rubisco synthesis in chloroplasts may be some light-mediated signal. Phytochromes make a good candidate as a major signalling mechanism for light-induced protein synthesis when they are transported to the nucleus upon photostimulation (Spalding and Folta, 2005). Indeed phytochrome-interacting factor 1 (PIF1) has been implicated in the greening of seedling plants, with PIF1 mutants accumulating a chlorophyll precursor which renders seedlings sensitive to light stress (Huq et al., 2004).

Carbohydrate accumulation, especially glucose, represses the transcript abundances of several photosynthetic genes, including Rubisco. Indeed, sugar levels are well known to regulate transcription (Sheen, 1990). Recent evidence has shown a negative correlation between leaf glucose levels and *rbcS* transcript abundances, in both wild-type and ethylene-insensitive *Nicotiana* plants (Acevedo-Hernandez et al., 2005). Acevedo-Hernandez's study exposed the cells to a range of sugar concentrations, noting that higher glucose availabilities rescued *rbcS* transcription after the initial reduction, implying multiple promoters and repressors, operating differentially under varying conditions. Interestingly, in the absence of glucose, abscisic acid (ABA) had a negative effect on *rbcS* transcript abundance, but a positive effect in the presence of glucose. Older studies, however, suggest that it is an intermediate in carbohydrate metabolism, rather than carbohydrate accumulation *per se* which controls *rbcS* levels (Krapp et al., 1991, 1993). Although transcription is generally thought to be more important in

determining mRNA levels, the importance of mRNA turnover should not be underestimated, with this being an important yet poorly understood factor allowing nuclear control of plastid genes (Drager et al., 1998). Other studies have shown that carbohydrate status is important in regulating chloroplast biogenesis itself, with *Arabidopsis* grown with supplemental glucose failing to green up and exhibiting significantly decreased levels of hexadecatrienoic acid—a major constituent of chloroplast membranes (To et al., 2003).

Similarly with ABA, cytokinins are also able to modify transcript levels, specifically upregulating them in barley leaves (Zubo et al., 2008). As discussed in more detail later, cytokinins are also able to retard proteolytic rates, while ABA has been shown to promote senescence. Zubo et al. (2008) used detached barley leaves in their experiments, showing that cytokinin application had the strongest effect on the apical, and therefore oldest, cells. Several genes were upregulated in response to exogenous cytokinin application, including the *rbcL* gene; however, interestingly the *psbA* and *psbD* genes, encoding the photosystem II (PSII) D1 and D2 proteins, respectively, were unaffected by cytokinin. Light appeared to be required for gene upregulation by cytokinins (Zubo et al., 2008). Nitrate supply to maize roots has been shown to lead to cytokinin transport to the leaves, which may act as a long-range signalling mechanism of N sufficiency (Sakakibara et al., 1998).

Atmospheric CO_2 concentration appears to have a strong influence on *rbcS* gene expression, with increased transcript abundances noted at low CO_2 levels (Gesch et al., 1998). Rice cultivars grown under elevated CO_2 levels and temperatures showed strong genotype-dependent changes in *rbcS* transcript abundance (Gesch et al., 2003). The CO_2-mediated decrease in *rbcS* transcript was temperature-dependent; one variety showed a CO_2-dependent decrease in *rbcS* only at 28°C, and not at either 34°C or 40°C, while the second variety exhibited a decrease at 34°C and 40°C, but not 28°C. Increased temperature caused a decrease in transcript abundance at 350 ppm CO_2, but not in plants grown at 700 ppm CO_2 (Gesch et al., 2003). Other studies have similarly shown a decrease in leaf Rubisco concentration by both elevated CO_2 and temperature in both rice and soybean (Vu et al., 1997).The mechanism by which these decreases are affected was unclear, whether due to a decrease in Rubisco synthesis or due to an increase in degradation. Increased photosynthesis at elevated CO_2 causes substantial increase in leaf sugar concentrations, especially sucrose and hexose (Vu et al., 2001), and sugars have been linked to increased proteolytic rates (Wingler et al., 2006).

However, C status is only one part of the puzzle, with N status also having important implications for gene expression (Scheible et al., 2004) and,

ultimately, the levels of photosynthetic enzymes within plant leaves (Matt et al., 2002). Increasing N availability leads to increased leaf Rubisco contents in hydroponically grown rice plants (Makino et al., 1984b); however, it is less clear how this relates to cellular Rubisco contents because plants grown at higher N levels also have larger leaves and larger cells. Thus, changes in leaf Rubisco concentration may be buffered at intermediate N levels due to changes in leaf size and thickness. Scheible et al. (2004) showed that the supply of N to N-starved *Arabidopsis* seedlings upregulated genes involved in chlorophyll biosynthesis, the photosynthetic light reactions and also numerous genes associated with the Calvin cycle and starch biosynthesis.

III. PROTEIN DEGRADATION

A. CELLULAR REGULATION OF PROTEIN DEGRADATION

In many eukaryotic cells, specific protein degradation in the cytosol and the nucleus is carried out in a ubiquitin-dependent pathway by the 26S proteosome. Ubiquitin conjugates with the protein to be degraded, which is then degraded by serine and cysteine proteases (CPs) in the proteosome (Buchanan-Wollaston et al., 2005).

Protein degradation is generally considered to occur in a stepwise manner, with an initial rate-limiting cleavage by a specific enzyme followed by further degradation of the cleavage products by generalist proteases. Although the general assumption is of a single protease being the rate-limiting step, the degradation of a single protein may be regulated by multiple pathways (Callis, 1995). Protein degradation is generally an ATP-dependent process, despite being an exogonic reaction, which could theoretically proceed without the requirement for energy (ATP) supply.

Leaf senescence is well regulated, with individual cells senescing in a well-defined order. In grasses, senescence primarily starts from the tip, the oldest cells, working towards the base of the leaf, with the cells around the leaf veins taking longest to senesce (Wingler et al., 2004). In *Arabidopsis*, similarly, a well-defined pattern of cell senescence can be seen, with cells around the veins taking longest to senesce, with similar patterns emerging in rice coleoptiles (Inada et al., 1998a, b). Since nutrient export from the leaves is dependent on the transfer of nutrients to new leaves through the phloem, cells bordering the veins would be expected to senesce last. An additional factor may be that cells bordering the phloem are more able to export C than those further away, since increased sugar levels in the cells can cause

senescence. Cells further away from the phloem may build up sugars more rapidly, promoting increased rates of senescence. However, as far as we are aware, this possibility has not been explored in detail, but would be interesting.

Senescence timing and characteristics can vary greatly depending on the environmental conditions under which the plant lives. For example, Wingler et al. (2004) grew *Arabidopsis* plants on agar with varying levels of N and glucose. Plants growing at reduced N supply visibly accumulated anthocyanins—plant pigments expressed under light stress conditions, which appear to function as a photosynthetic "light shield" (Albert et al., 2009). Non-photosynthetic quenching (NPQ) was shown to increase during senescence, while the dark-adapted fluorescence yield (Fv/Fm) was relatively unaffected. This tells us that while chlorophyll levels are remaining relatively stable, a smaller fraction of the incident energy is being used to drive photochemistry. Increased NPQ is indicative of light stress, while the liberation of free-chlorophyll from the thylakoid membranes can drive the production of free-radicals of oxygen. Oxygen radicals are hugely dangerous for the cells, and complex biochemical pathways exist to mitigate their production (Asada, 2006). These mechanisms are not 100% effective, however, and the chloroplast becomes a more oxidizing environment through time (McRae and Thompson, 1983). We might hypothesize that these increases in active oxygen levels cause the vacuole rupture which precedes cell necrosis (Obara et al., 2001), although this is unknown. While the addition of glucose to low-N media leads to an increase in the rate of leaf senescence in *Arabidopsis*, plants grown on high-N media with supplemental glucose did not exhibit accelerated senescence and were dark green than the non-glucose supplemented plants (Wingler et al., 2004).

Many genes coding for protein kinases and phosphatases, along with several genes involved in calcium metabolism, are upregulated during senescence, indicating the functioning of kinase-signalling cascades (Buchanan-Wollaston et al., 2005). There is some evidence linking increased calcium levels with cell death (Huang et al., 1997), with calcium being necessary for the functioning of some CPs (Callis, 1995). A vast array of genes involved in N metabolism and sugar, peptide, amino acid and cation membrane transporters are also upregulated. Transcript abundances for genes involved in glutamate/glutamine metabolism are upregulated, including glutamate receptor proteins and glutamine synthetase (GS) (Buchanan-Wollaston et al., 2005). Several genes involved in ABA signalling were upregulated, while various cytokinin-induced genes were downregulated. This suggests an increase in ABA levels and a decrease in cytokinin levels in senescing leaves. Exogenously supplied cytokinins have been shown to retard chlorophyll loss

in excised *Arabidopsis* leaves (Sergiev et al., 2007), and cytokinin decrease has been hypothesized to be a major factor in shade-induced senescence (Causin et al., 2009). Similarly, a maize stay-green mutant exhibited increased levels of leaf cytokinins, while ABA levels were decreased compared to an early senescent variety (He et al., 2005). Both cytokinin and ABA have been shown to have effects on leaf sugar regulation (Yang et al., 2002), with one study suggesting that a mutant's stay-green phenotype resulted from decreased C transport from leaves (Yang et al., 2003) although it is unclear how much of the extra remobilization and transfer of stored C is directly hormonally mediated, and how much is simply a result of decreased amounts of recent photosynthate as ABA causes stomatal closure (Acharya and Assmann, 2009). Recent photoassimilate is generally important in supplying C for growth (Lattanzi et al., 2005), with photosynthesis expressed per unit plant mass and relative growth rate showing a strong linear relationship (Kruger and Volin, 2006).

During starvation-induced senescence in cell suspension culture, many of the cytokinin-inducible genes downregulated under natural senescence were not expressed at all, while little change from control conditions could be seen under dark conditions. Since cytokinins tend to retard senescence, the absence of cytokinin-inducible genes presumably reflects an absence of cytokinins and increased proteolytic rates. Cytokinins appear to be important in dark-induced senescence; however, the mechanism of dark-induced senescence is unclear, as the expression of cytokinin-induced genes is quite dissimilar to that in control of C-starved conditions (Buchanan-Wollaston et al., 2005).

Various other hormones have been reported to be linked to leaf senescence, and transcriptomic approaches have demonstrated increases in transcripts related to, for example, jasmonic acid and salicylic acid metabolism (Van der Graaff et al., 2006). Jasmonic acid has been shown to increase over the period of senescence in the leaf (He et al., 2002), but like ABA both jasmonic acid and salicylic acid also accumulate during stomatal closure, and have been linked to that (Acharya and Assmann, 2009). On the other hand, there are some suggestions that senescence-associated genes (SAGs) are dependent on salicylic acid (Van der Graaff et al., 2006). Finally, two other classes of compounds, gibberellins and brassinosteroids, were identified by Van der Graaff et al. (2006) as potential hormones involved in senescence, despite direct evidence being relatively scarce for these two chemicals. Interestingly, along with all the other hormones discussed above, both gibberellins and brassinosteroids were identified by Acharya and Assmann (2009) as being involved in determining stomatal aperture. Thus, the exact relationship between stomatal aperture and senescence

remains unclear, although it is clear that a relationship exists. Ethylene is understood to be important in determining the rates of leaf senescence, with ethylene-insensitive mutants exhibiting increased leaf lifespan, including an increased duration of protein retention, compared to control plants (Grbic and Bleecker, 1995). Ethylene was also shown to increase the transcript abundances of SAGs, although the effects of ethylene were more pronounced in old leaves than in younger leaves, suggesting age-dependent effects. However, like the control plants, ethylene-insensitive plants also upregulated SAGs in senescent leaves, although this was delayed compared to control plants (Grbic and Bleecker, 1995). This suggests rather that ethylene has a role in moderating the timing of leaf senescence, although ultimately the leaves senesced in both control and ethylene-insensitive varieties.

The majority of proteolysis takes place in the vacuole, due to its low pH (Bassham, 2009), with both proteases (Yoshida and Minamikawa, 1996) and protein degradation products (Huffaker, 1990) found there. Proteins from the cytosol or chloroplasts must be taken into the vacuole by the process of autophagy (ATG). ATG was originally characterized in yeast (Tsukada and Ohsumi, 1993), but many ATG genes have also been identified in plants, especially *Arabidopsis* (Hanaoka et al., 2002; Inoue et al., 2006), but also in maize (Chung et al., 2009). Recently, Van der Graaff et al. (2006) noted that 19 out of 21 identified ATG genes were upregulated during natural senescence.

Genes for cytosolic GS were not strongly expressed in dark-mediated senescence. These genes generally fix ammonia produced during photorespiration, which can be assumed to be negligible under dark conditions. However, other N-related genes are upregulated; for example, various transaminases are increased under dark-induced senescence (Buchanan-Wollaston et al., 2005).

B. PLANT PROTEASES

Plant cell endoproteases can be divided into four main groups: CPs, serine proteases, metalloproteases and aspartate proteases (Callis, 1995). A fifth group, consisting of those which cannot be identified as one of the above four, also exists.

CPs generally have an acidic pH optimum and are generally located in the vacuole. However, as mentioned above, CPs have also been implicated in regulating protein degradation of chloroplast stromal constituents (Minamikawa et al., 2001; Prins et al., 2008), although the specific location

of degradation remains unclear. Certainly, the stromal pH should be too high for CPs to work efficiently.

As mentioned previously, the 26S proteosome is a proteolytic complex found in the cytosol and nucleus of eukaryotic cells. The proteosome is a complex of generic proteases, which tend to have a neutral pH. Prior to degradation by the proteosome, ubiquitination of target proteins is required. The attachment of ubiquitin to the protein is an ATP-dependent process (Demartino and Slaughter, 1993) achieved through the action of three enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase (E3) (Moon et al., 2004). E3 protein ligases are substrate-specific, and over 90% of the approximately 1400 genes involved in the ubiquitin proteosome pathway code for E3 protein ligases (Smalle and Vierstra, 2004). Phytochrome-mediated light signals have been demonstrated to increase the activity of some E3 protein ligases, which may have an important role in photoacclimation or other regulatory processes (Huq, 2006). The proteosome is generally considered important in the cell cycle, with mutation in 26S proteosome genes preventing successful mitosis (Ghislain et al., 1993; Gordon et al., 1993). Genes involved in the ubiquitination of proteins for degradation were not greatly affected by the resupply of N to N-deficient *Arabidopsis* seedlings, a finding which may be surprising given that we might expect comprehensive changes in plant growth strategy under N-deficient and N-sufficient conditions (Scheible et al., 2004).

Various stresses may also upregulate protease expression. For example, osmotic and salt stress has been shown to lead to an increase in mRNA transcript abundances, although this was not related to abscissic acid levels (Koizumi et al., 1993). CP mRNA levels increased in cold-treated tomato fruits (Schaffer and Fischer, 1988), while heat shock is well known to induce proteolytic activity in bacterial and yeast cells (Finley et al., 1987; Katz et al., 2009). Oxidative and UV-B stress leads to Rubisco fragmentation (Desimone et al., 1996; Ishida et al., 1997, 1998); however, these fragments accumulate to levels detectable by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), suggesting that either protease levels in the chloroplast or the cell or the rate of transport of the fragments to the site of degradation is insufficient to degrade the fragments before they start to build up. It seems likely that these fragments would be in a proteolytically susceptible state, implying that even the generic proteases which would normally be involved in fragment degradation are limiting. Oxidative modification is known to enhance the binding of Rubisco protein to the chloroplast envelope (Marin-Navarro and Moreno, 2006), presumably making vesicular transport from the chloroplasts to the vacuole by Rubisco-containing bodies

(RCBs) (discussed below) more likely. Other recent work has shown that oxidative stress can lead to an upregulation of macroautophagy in *Arabidopsis*, presumably as a mechanism to deal with oxidatively damaged proteins.

RNA is perhaps particularly susceptible to degradation by UV-B, which may limit the *de novo* synthesis of proteases under these conditions (Takeuchi et al., 2002). Proteolysis is known to be important in seed germination, and often the proteases involved were synthesized during seed filling and drying. It is tempting to hypothesize that the production of these proteases is upregulated by water stress during seed desiccation.

Vacuolar proteases have been shown to be able to degrade the Rubisco large, but not small, subunit (Yoshida and Minamikawa, 1996). This has led to the speculation that the majority of proteolysis must occur in the vacuole, with proteins being transported from other cellular locations into the vacuole. This will be discussed in more detail below, however, it should be pointed out here that the fact that the vacuole contains proteases, which are capable of degrading Rubisco, does not mean that Rubisco degradation is their primary, or even normal, function—many general proteases exist, able to degrade a wide range of proteins. Small subunit degradation is relatively understudied, although some reports exist (Miller and Huffaker, 1982). Miller and Huffaker used ^{14}C-labelling of Rubisco and found three endoproteases with the ability to degrade the Rubisco, naming them EP1, EP2 and EP3. EP1 is a chloroplast-located metalloprotease, which primarily acts upon the RBCL. EP1 degrades Rubisco to a 36-kDa fragment *in vitro*, and it seems likely that EP1 also acts in a similar manner *in vivo*. EP2 primarily acted against the small subunits, while EP3 caused the autolysis of Rubisco purified by SDS–PAGE.

Unassembled proteins, or those with mistakes, tend to be rapidly degraded *in vivo*, with unassembled 32S-labelled yeast fatty acid synthase α-subunit being almost completely turned over within 6 hr after the end of the labelling period (Baek and Choi, 2008), although that is outside the scope of this chapter.

C. CHLOROPLAST STROMAL PROTEIN DEGRADATION

As leaves senescence, the protein concentration of the individual cells decreases. Early studies showed that protein concentration decreased at a much higher rate than the loss of chloroplasts within cells (Mae et al., 1984; Wardley et al., 1984). This suggests that chloroplast loss itself cannot be solely responsible for the noted decrease in leaf Rubisco concentration through time, suggesting that Rubisco and other chloroplast stromal

proteins must either be degraded within the chloroplast or be exported from the chloroplast then degraded. Chloroplast protein fragments have been isolated in the vacuole (Huffaker, 1990), implying that the vacuole is a major site of proteolysis in plants, and it is known that wheat chloroplasts shrink through time (Ono et al., 1995). Recent studies have shown chloroplasts budding off small spherical Rubisco-containing vesicles, dubbed RCBs, which are then degraded within the vacuole (Chiba et al., 2003; Ishida et al., 2008; Wada et al., 2009). While RCBs were named based on their concentration of Rubisco, it is clear that they also contain other stromal proteins, for example, Gln synthetase, or chloroplast-targeted green fluorescent protein (GFP) in its native form (Ishida et al., 2008), which may imply a lack of specificity for stromal proteins. RCBs do not, however, contain thylakoid membranes, with Wada et al. (2009) noting that chlorophyll was absent from the fluorescence emission spectra of RCBs; however, it is unclear whether the degradation products of thylakoid-associated proteins are transported in RCBs. However, immunolocalization studies failed to show any signal when RCBs were stained with antibodies for thylakoid proteins, such as LHCII or cytochrome (Chiba et al., 2003). In ATG-deficient mutant *Arabidopsis* plants, which lack the capacity to form RCBs, the rate of Rubisco degradation was unchanged, while chlorophyll catabolism was increased in the mutants (Wada et al., 2009). This implies the existence of other mechanisms of Rubisco degradation, probably stromal proteases, and may even suggest that RCB production is incidental and unnecessary for Rubisco degradation. On the other hand, Wada et al. (2009) showed the build up of GFP-labelled stromal proteins in the vacuoles of darkened control plant leaves treated with concanamycin A, which inhibits vacuolar ATPase causing a pH shift repressing protease activity. This clearly suggests that stromal proteins are transported to the vacuole without the breakdown of GFP, and presumably other proteins also. Furthermore, the increased chlorophyll catabolism in the *atg4s* mutant leaves suggests elevated protease levels in the chloroplasts of these plants compared to control plants, further implying that the breakdown mechanism in the *atg* mutants is not the normal degradation pathway. The *atg* genes were discovered initially in yeast; however, they were later noted in *Arabidopsis* and appear to be important in the production of RCBs. The *atg* mutants used in Ishida et al. (2008) exhibit increased stromule activity. Stromules are stroma-filled protrusions of the chloroplast envelope, although their function is unclear (Holzinger et al., 2007). Interestingly, the rate of proteolysis is identical whether RCBs can be formed or not, which suggests that the rate of Rubisco degradation may be controlled by some factor preceding either export by RCBs or degradation by chloroplast proteases.

The structure and function of stromules remains unclear, although various functions have been postulated. However, as chloroplasts are derived from ancient free-living bacteria which formed an endosymbiotic relationship with proto-plant cells (Sagan, 1967), and bacteria routinely transfer genetic information in the process of conjugation, stromules may be derived from this process. It seems plausible that RCBs could be derived from stromule protuberances, with RCB-sized vesicles being pinched off from stromules noted previously (Arimura et al., 2001; Waters et al., 2004; Gunning, 2005). Furthermore, similar to RCBs, these stroma-containing vesicles do not contain thylakoid material (Holzinger et al., 2007).

RCB production within leaves is known to vary over time (Chiba et al., 2003). In Ishida et al. (2008), the number of RCBs in the vacuole of concanamycin A-treated cells was noted for the fourth, sixth, eighth and tenth leaves of *Arabidopsis*. While RCB number was low in the youngest leaves (10th) showing high *rbcS* 2B gene expression, which suggests active Rubisco synthesis, RCBs were present suggesting some turnover even in expanding leaves. RCB number increased approximately fourfold in the eighth leaf, then a further twofold in the sixth leaf. RCB numbers were approximately similar in the fourth and sixth leaves, although variability in the number of RCBs in the fourth leaves was higher (Ishida et al., 2008). This pattern of RCB numbers correlates with the rates of Rubisco throughout the leaves' lifespan, which is considered to be relatively low during leaf expansion, increasing around full leaf expansion and then generally high during senescence. Factors controlling RCB production remain unknown, yet these patterns of RCB production through time suggest strong ontogenic effects, although it is unclear whether these are genetic or environmental. RCB studies are generally conducted on darkened leaves and it is difficult to visualize RCBs in leaves which have not been incubated in the dark. Light acts both as the energy source for photosynthesis and via photoreceptor chemicals such as phytochrome as a signal in its own right. It is unclear, therefore, whether RCBs are produced to remobilize N from shaded leaves or RCB formation is controlled by leaf sugar status. Certainly, it is known that whole-darkened plants do not senesce (Weaver and Amasino, 2001). These plants similarly do not grow, so the lack of proteolysis may be due to either a reduction in the sink strength of developing leaves or a lack of C derived under normal circumstances from other leaves.

ATG of whole chloroplasts takes place predominantly close to the end of the leaves lifespan. Wada et al. (2009) showed an increased rate of chloroplast ATG in darkened *Arabidopsis* leaves. The *Arabidopsis* plants used had been bred with chloroplast–stroma-targeted GFP, allowing the visualization of RCBs by laser-scanning confocal microscopy (Fig. 1). While RCBs

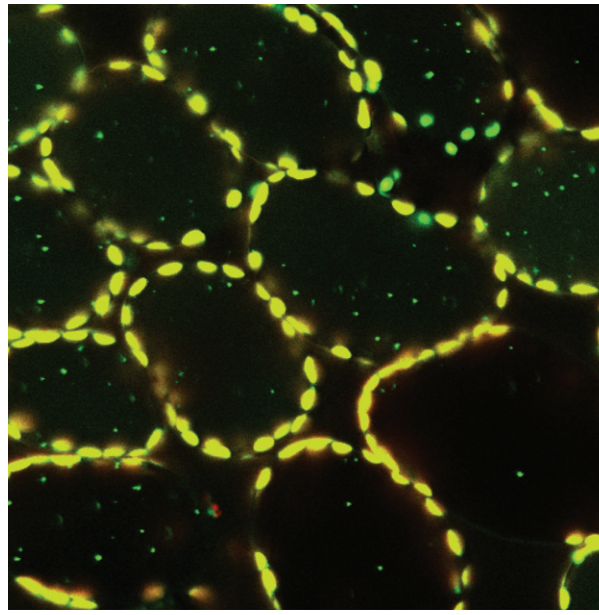

Fig. 1. Visualization of RCBs in living leaf cells of *Arabidopsis* by stromal-targeted GFP and laser-scanning confocal microscopy. Stromal-targeted GFP is shown by pseudo green and chlorophyll fluorescence is by pseudo-red and the merged image is shown. Chloroplasts having both GFP and chlorophyll appear yellow, whereas RCBs having only GFP appear green. (See Color Insert.)

strongly exhibited the GFP signal, vacuolar-located chloroplasts exhibited fluorescence signals associated with chlorophyll fluorescence only, with no stromal proteins apparent. It is unclear how much stromal protein the chloroplasts contained at the time of transport, or whether stromal proteins are simply more proteolytically susceptible and were degraded upon the chloroplast being subsumed. There are some reports that the DNA content of chloroplasts decreases at a relatively early point during senescence, preceding large-scale proteolysis (Inada et al., 1999).

Small Rubisco-containing vesicles have been noted by other investigators exploring senescence in tobacco, although reported as "senescence-associated vacuoles" (SAVs) (Martinez et al., 2008; Otegui et al., 2005). SAVs were noted to have a pH lower than the central vacuole, which in turn is lower than chloroplast pH values (Laisk et al., 1989; Oja et al., 1999). Martinez et al. (2008) used transformed tobacco plants with chloroplast-targeted cyan fluorescent protein (CFP). Leaves were induced to senesce and produce SAVs by detachment from the plant and incubation in continuous darkness and it is unclear in Martinez et al.'s (2008) study whether SAVs could be visualized

under normal conditions; however, RCBs cannot be seen under control conditions, so it is unclear whether SAVs can be visualized under more normal conditions. However, contrary to the situation in RCBs, Martinez et al. (2008) noted that some tobacco SAVs exhibited a chlorophyll fluorescence signal; however, they did not note this in either *Arabidopsis* or soybean in an earlier study (Otegui et al., 2005). This may confirm either that RCBs and SAVs are indeed separate and independent organelles, or that the inclusion of chlorophyll in RCBs is species-specific, or that chlorophyll degradation products were found in true RCBs by Martinez et al. (2008). Dark-incubation, carbohydrate starvation and natural senescence all seem to be separate processes, with very different transcript profiles for each, and it is possible that either RCB can contain chlorophyll under these conditions or SAVs are unique vacuoles associated with a wounding response to the leaf detachment. Interestingly, Martinez et al. (2008) noted that dark-incubated, excised tobacco leaves treated with ethylene produced approximately double the number of SAVs than control leaves, untreated with ethylene. The percentage of these containing the GFP signal was approximately the same as in the control plants, and it was suggested that this indicates a higher rate of degradation. It is unclear exactly how quantitative this work is though, and doubling the number of SAVs will not lead to a greater rate of protein degradation if the protein concentration in those SAVs is halved.

Martinez et al. (2008) also noted high levels of chloroplastic GS2 in isolated SAVs as well as large quantities of Rubisco. Furthermore, they demonstrated the presence of proteases inside isolated SAVs, by showing that the addition of protease inhibitors in the culture media retarded the degradation of the RBCL. Martinez et al. (2008) went on to conclude that since SAVs contain both proteases and chloroplast proteins, they have an important role in mediating the loss of chloroplast proteins in tobacco. However, significantly, they failed to quantify the rates of protein loss from the chloroplasts or to provide evidence that SAVs have sufficient protease activity to achieve noted rates of protein degradation. In Wada et al.'s (2009) study, plants unable to produce RCBs suffered no retardation in their ability to degrade Rubisco—degradation rates were identical in control and RCB-less plants—suggesting that RCBs, and potentially SAVs too, are unnecessary to explain Rubisco degradation in plant leaves. Interestingly, Wada et al. (2009) did not note any small spherical bodies in ATG-deficient (*atg*) mutant plants, nor any accumulation of stromal-targeted GFP in the vacuoles of concanamycin A-treated mutants, suggesting either that SAVs are not present in *Arabidopsis* or that SAV production is similarly dependent on the ATG gene, or that protein transported in SAVs is degraded *en route* to the vacuole. For SAVs and RCBs to be both ATG5 dependent would be a huge coincidence, which while not obviously silly, seems statistically implausible. However, both Martinez et al.'s (2008)

evidence of chlorophyll constituents in SAVs and Wada et al.'s (2009) evidence of the lack of chlorophyll fluorescence in RCBs are troublesome, and it is hard to reconcile these apparently contradictory reports other than by invoking the somewhat unsatisfying answer of "species-specificity". SAVs differ from RCBs in a second key way—while RCBs exhibit a double-membrane structure, SAVs exhibit only a single membrane. The reason for this is again unclear, although it may be that while RCBs are enclosed in a second membrane, transported to the vacuole and assimilated in macroautophagy, SAVs may undergo microautophagy and be assimilated directly into the vacuole. If this is true, it may suggest that SAVs and RCBs are in fact the same structures, with Martinez et al. (2008) simply noting either chlorophyll breakdown products in the vacuoles or the sensitivity of their HPLC approach simply being far higher than the sensitivity of Wada et al.'s (2009) chlorophyll fluorescence approach. As Martinez et al. (2008) did not quantify the proteins noted, it is difficult to differentiate between these possibilities. However, while the chlorophyll signals noted in Martinez et al.'s (2008) paper (Fig. 3b in Martinez at al. (2008)) are rather weak, the GFP signal is much stronger, and it is difficult to see any blue colouration in the merged image. Furthermore, no chlorophyll fluorescence can be noted in a SAV-enriched fraction.

Isolated chloroplasts cannot degrade Rubisco completely, with only the initial cleavage of the RBCL occurring, yielding a 44-kDa fragment (Kokubun et al. 2002). Wada et al.'s (2009) data, however, clearly showed that RCBs are unnecessary for Rubisco degradation. This implies that Rubisco is not exported from the chloroplast, even by leakage or membrane transport, simply since we do not see leakage of GFP from the chloroplast, but instead that Rubisco (and GFP, and presumably other proteins) is degraded within the chloroplast. These two pieces of information taken together, the inability of isolated chloroplasts to degrade Rubisco coupled with the degradation of Rubisco within the chloroplast in mutants unable to produce RCBs, suggest a cytoplasmic origin of the majority of Rubisco proteases, agreeing with the schemes of both Prins et al. (2008) and Martinez et al. (2008). Isolated chloroplasts, treated with thermolysin to degrade contaminating proteases adhering to the chloroplast envelope, displayed the ability to degrade the RBCL, in a manner that was time-, temperature- and pH-dependent (Zhang et al., 2007). Contrary to Kokubun et al.'s (2002) data, Zhang et al. (2007) noted that the initial cleavage product was a 51-kDa fragment, although this may have been a result of the chloroplasts being dark induced to senesce. Zhang et al. (2007) noted that the cleavage site of their noted 51-kDa fragment was similar to that for a previously noted vacuolar protease. Since the experiment was conducted using isolated

chloroplasts, the presence of a vacuolar protease seems strange; however, it seems plausible that some proteases may be transported along with Rubisco and other proteins from the chloroplast to the vacuole in RCBs, where the proteases, but not the Rubisco, accumulate. Zhang et al.'s (2007) data further suggested that the vacuole is an unlikely venue for at least the initial few cleavages of the Rubisco subunits, although it seems likely that the later stages of degradation occur there.

Recent work has illustrated the importance of CPs in the degradation of chloroplast proteins, including Rubisco and Rubisco activase (Minamikawa et al., 2001; Prins et al., 2008). Minamikawa et al. (2001) used antibodies raised against Rubisco and a CP in detached French bean leaves, noting that over the 8-day incubation period, chloroplasts were taken into the vacuole and degraded. Prins et al. (2008) overexpressed a rice cystatin in tobacco plants, which blocks the protease action of the CPs, leading to significantly higher leaf protein concentrations, and commensurately higher photosynthetic rates, than control tobacco leaves, apparently due to a decrease in proteolysis. Unfortunately, since protein levels were not been quantified through time for individual leaves, it is unclear whether these increases in protein concentration represent the steady increase in leaf protein through time, or whether the majority of the proteins were produced during leaf expansion, with relatively little subsequent protein synthesis. Repression of CPs by cystatin led to an increase in total CP activity, which may suggest that CP expression and activity may be modulated by amino acid or protein levels. While the major protein degradation site is postulated to be the vacuole, cystatin is largely found in the cytosol, although was also found in the chloroplasts and vacuole.

D. THYLAKOID-ASSOCIATED PROTEINS

Other proteins, for example, those associated with the thylakoid membranes do not appear to be exported from the chloroplast and degraded, but rather to be degraded *in situ*. The D1 protein may have one of the shortest half-lives of any protein in any living system and may be the most researched. The D1 protein forms a complex with the homologous D2 protein—both of which have a molecular mass of approximately 32 kDa. The D1–D2 complex forms a part of the PSII reaction centre. During illumination, the D1 complex is degraded, although the specific mechanism by which this occurs is disputed. Older literature with much current literature argues that D1 is initially cleaved by the action of oxygen radicals on the protein. This would seem to be borne out by evidence showing that D1 degradation rate is strongly dependent on light intensity, with degradation rates increasing rapidly

between 0 and 250 μmol photons m^{-2} sec^{-1}, and more slowly thereafter (Edelman and Mattoo, 2008). However, it does raise the question of why D1 degradation is not progressively faster at higher irradiances. Other, more recent, investigations have raised alternate possibilities, such as the hypothesis that the primary action of reactive oxygen species is in inhibiting D1 synthesis, which negatively affects the repair cycle of D1 (Takahashi and Murata, 2008). Treatment with propyl gallate, a reactive oxygen species scavenger, has been reported to promote D1 synthesis (Edelman and Mattoo, 2008). Suitably high light will also cause damage to the D2 protein, which is also degraded under high levels of photo-oxidative stress. Unlike the D1 protein, however, two beta-carotene molecules bind to the D2 protein, quenching oxygen radicals, and protecting D2 from degradation (Telfer, 2005). Proteolysis of the D1 protein appears to follow a structural modification to the protein structure, possibly a covalent modification (Andersson and Aro, 1997). Although this conformational modification is generally light-dependent, detergent treatment of the PSII complex may induce D1 protein degradation in darkness (Nakajima et al., 1995, 1996). Similarly, the use of a PSII inhibitor, PNO8 (N-octyl-3-nitro-2,4,6-trihydroxybenzamide) facilitated the degradation of the D1 protein in darkness. Furthermore, in *Chlamydomonas*, when a conformational change in D1 is suppressed by the interaction of the PQH_2 ligand with the Q_B site of D1, the subsequent degradation of D1 is also prevented (Zer et al., 1994). These data, taken together, suggest that the enzymes required for the degradation of D1 are available at all times; however, the conditions for the conformational change under normal conditions generally only occur in the daytime, under high light conditions. Recent evidence has suggested a key role for the FtsH proteases in the turnover of the D1 protein (Kato et al., 2009), although it is unclear whether FtsH causes the primary cleavage of the D1 protein, or whether it is more important in its subsequent degradation. Mutant plants lacking the FtsH protease tend to accumulate higher levels of oxygen radicals than wild-type plants.

ATP-dependent post-transcriptional phosphorylation appeared to be a second mechanism regulating the degradation of the D1 protein in higher plants (Kettunen et al., 1991); however, later studies, in which the breakdown of unphosphorylated control D1 protein and D1 protein which had been phosphorylated by exogenous addition of [γ-^{32}P]ATP, showed that phosphorylated D1 degradation occurs at a lower rate than unphosphorylated D1 (Andersson and Aro, 1997).

Plants grown under low light conditions produce a greater quantity of the LHCII protein than those grown under high light. LHCII is a protein involved in the capture of photons during photosynthesis and is the most

abundant thylakoid protein. At high photon flux densities, high levels of LHCII are unnecessary and presumably would lead to increased light stress. Thus, after a lag period of approximately 2 days, chloroplast LHCII contents are decreased. Thus it appears to be effected by the degradation of the outermost LHCII proteins, by an as-yet unknown ATP-dependent cysteine or serine protease (Yang et al., 1998). The rate of proteolysis is substrate limited. Furthermore, like the D1 protein discussed above, phosphorylation appears important in determining the rate of LHCII degradation with phosphorylated LHCII being a poor substrate for degradation, similar to the D1 protein. The major site for the degradation of thylakoid proteins appears to be the stroma-exposed membrane regions, rather than the appressed regions, where thylakoid membranes are "stacked" one on top of the other. This suggests that thylakoid protein degradation may be facilitated by stromal proteases. It is known, however, that neither Clp nor FtsH are responsible for the degradation of LHCII (Yang et al., 1998).

E. ENVIRONMENTAL REGULATION OF SENESCENCE

Various environmental factors are well known to regulate the rate of leaf senescence, with many signalling pathways leading to chlorosis. For example, sugars are understood to be implicit in the process, with high leaf sugar levels promoting protein degradation and leaf senescence (Wingler et al., 2006). Young expanding leaves are C deficient, as the processes of cell division, expansion and protein synthesis require large amounts of energy. However, as leaf expansion completes, and the chloroplasts mature, we would expect the leaf's C budget to shift from being negative to positive, and for the leaf to accumulate sugars and become a net C source. Indeed, sugar contents of *Arabidopsis* leaves increase through time, with additional glucose further increasing the apparent rate of senescence, as determined by reductions in Fv/Fm (Wingler et al., 2006).

Cold treatment can delay senescence, even despite the accumulation of sugars and ABA, factors which normally promote senescence (Masclaux-Daubresse et al., 2007). The reasons for the accumulation of sugars in cold-treated plants are not well understood; however, it could simply be related to lower growth and respiration rates at reduced temperatures. Certainly, the cold-grown plants shown in Fig. 2A of Masclaux-Daubresse et al. (2007) were much smaller than the control plants. Reduced growth rates would almost certainly lead to a large divergence from the plants' normal source—sink ratio. In some ways, this may be similar to the whole-darkened plants noted above, which do not grow, and do not show visible symptoms of senescence.

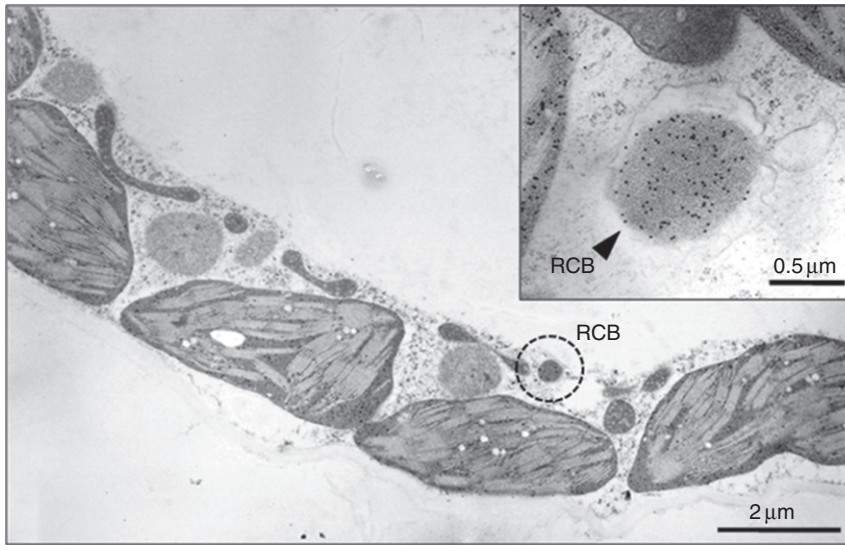

Fig. 2. Detection of RCB in naturally senescing wheat leaves by immunoelectron microscopy. Gold particles bind to RBCL (black spots). RCBs seem to be surrounded by autophagosome-like membrane structures.

Both water deficit and waterlogging can lead to premature senescence (Irving et al., 2007; Yang et al., 2002). Yang et al. (2003) noted that water-deficient wheat plants exhibited increased stem ABA, decreased cytokinin levels and increased rates of chlorophyll loss, when compared to water-sufficient control plants. While a direct hormonal role on protease levels or activity cannot be ruled out, it seems plausible that ABA effects are indirect. ABA plays an important role in regulating plant water status, causing stomatal closure under drought conditions (Acharya and Assmann, 2009), and has been directly implicated in the production of cellular reactive oxygen species (Zhang et al., 2001). Furthermore, stomatal closure may limit photosynthetic rates, promoting N loss from the leaf like ammonia produced during photorespiration, and also further causing the production of oxygen radicals. Cytokinins, conversely, promote stomatal aperture and decrease stomatal sensitivity to ABA, which may help to maintain photosynthetic rates, preventing the production of oxygen radicals and retarding senescence (Criado et al., 2009). Cytokinins may have a secondary effect, by promoting cell division and leaf growth, and causing an increase in protein synthesis. The process by which

waterlogging causes senescence remains unclear, but may be related to carbohydrate build up in the stem (Castonguay et al., 1993), leading to a sugar-based repression of photosynthesis (Araya et al., 2006) and increased light stress. Senescence may, ironically, proceed somewhat along the same lines in both water-deficient and waterlogged plants. Interestingly, suppression of drought-induced senescence by the overexpression of an isopentenyltransferase gene in tobacco led to a superior drought resistance, with the plants maintaining the ability to photosynthesize during the drought treatment, and even improved survival, with the transgenic plants surviving a drought which killed the control plants (Rivero et al., 2007). Conversely, root waterlogging may lead to a pH decrease in the root medium, with leaf damage caused by reduced leaf pH as water is transpired through the plant. Other stresses may be important in determining protein levels; for example, supplemental ozone supply has been demonstrated to decrease Rubisco transcript abundances, particularly *rbcL* levels (Glick et al., 1995). Similarly, water deficit has been shown to cause dose-dependent decreases in both Rubisco gene transcript abundances and Rubisco concentrations, and it is unclear whether Rubisco degradation rates were upregulated or the reduced protein levels were solely a result of suppressed protein synthesis (Bauer et al., 1997).

Many hundreds of SAGs have been identified over the years, although some of these have been identified under somewhat unnatural conditions, for example, in detached or dark-incubated leaves, and so some caution should be exercised (Buchanan-Wollaston et al., 2003; Chen et al., 2002; He et al., 2001). Developmental senescence (i.e. natural senescence) and starvation-induced senescence have been compared, and significant differences noted, particularly at the gene transcript level. Similarly, *Phaseolus* leaves when detached from the plant senescence more rapidly than attached leaves (Yoshida and Minamikawa, 1996). The physiological relevance of studies in which plants are placed under extreme condition such as these is questionable; however, because it is unclear whether the noted increases in senescence were related to leaf C balance, water content, light stress, were hormonally related, or for some other reason. Microarray analysis of transcript abundance in *Arabidopsis* leaves undergoing natural, starvation-induced or dark-induced senescence indicated profound differences in the number and identities of genes upregulated. While nearly 2000 genes were upregulated under dark-induced conditions, and nearly 1500 genes were upregulated under starvation-induced senescence, only 827 genes were upregulated under control conditions (Buchanan-Wollaston et al., 2005). This makes it likely that a huge number of SAGs, which have been identified under starvation- or

dark-induced senescence conditions, have no function in natural senescence, and may suggest that studies using dark-induced senescence have limited relevance when considering natural leaf senescence. Of course, the specific mechanisms causing the senescence of leaves under natural conditions remain unclear and may be the result of either genetic or environmental factors—further complicating our evaluation of the importance and the role of individual SAGs under any given conditions. Another study reported the upregulation of approximately 1300 genes during natural senescence in *Arabidopsis* (Van der Graaff et al., 2006). Van der Graaff et al. (2006) also reported that approximately 2000 genes were regulated during stress-induced senescence, although some of these 2000 must have been downregulated. The reasons for the differences between these two studies are hard to reconcile but may be related to the different growth and sampling conditions used in the two studies. Irrespective of these inconsistencies, it is clear that senescence is a genome-wide event, involving the action of many genes, in networks which are currently almost completely obscure.

Cell death is the final stage in the senescence of the leaf cell. During cell necrosis, the cell's vacuole lyses (Obara et al., 2001), lowering the pH of the cells and releasing vacuolar proteases which degrade the cellular constituents for remobilization to other organs.

IV. WHOLE-LEAF REGULATION OF PROTEIN CONTENT

However, regulation at the whole-leaf and whole-plant levels is more poorly understood, despite continued efforts at understanding the processes involved (Hidema et al., 1991; Imai et al., 2005, 2008; Ishizuka et al., 2004; Mae et al., 1983; Makino and Osmond, 1991; Makino et al., 1997b; 1984a, b; Suzuki et al., 2001, 2007). A recent paper developed a mathematical modelling process to help elucidate component processes and further understand the regulation of Rubisco synthesis at a whole-leaf level (Irving and Robinson, 2006).

Rubisco synthesis takes place in a physiologically well-defined zone, 50–100 mm from the site of cell division (Gastal and Nelson, 1994). According to the Irving and Robinson (2006) model, through time Rubisco synthesis rate approximates a normal curve, correlating with leaf expansion rates, while degradation follows the first-order kinetic rules; degradation rates are initially low during leaf expansion increasing to a maximum just after full leaf expansion when Rubisco concentration is maximal. This is

followed by a period of senescence at a steadily decreasing rate, as the degradation rate decreases with decreasing Rubisco concentration, through to leaf death. Thus, through time, Rubisco content can be described by a log-normal curve. Rubisco turnover, as defined here, represents the sum of activity of both the biosynthesis and degradation of Rubisco within the leaf (i.e. faster turnover equals more synthesis and degradation, slower turnover less).

A recent review expressed some scepticism over the validity of using a single decay constant in the Irving and Robinson (2006) model, citing the stabilizing effects of CA-1-P binding and Rubisco activation in preventing Rubisco degradation (Parry et al., 2008). This point of view may find some support in the data of Ishizuka et al. (2004), which suggest that Rubisco degradation rates are greatly decreased and cannot be described by a single decay constant. However, caution should be exercised in interpreting the data of Ishizuka et al. (2004) as they does not represent a true time course, in which they measured Rubisco contents in leaves at different positions, rather than making measurements on the same leaf of replicate plants through time. Furthermore, it is not clear whether Rubisco turnover kinetics is identical in all leaves, or whether the protein turnover rates change with plant age. Finally, we would point out that the degradation mechanism of Rubisco remains very unclear, we do not know, for example, whether Rubisco is primary degraded at night time or during the day, or whether it is continuous and invariant. Thus, it may be premature to attempt a quantitative argument for the importance or otherwise of Rubisco activation or inhibitory compound on Rubisco turnover rates. Irrespective of the logic of Parry et al. (2008) that CA-1-P and other compounds *should* lead to a variable rate of Rubisco catalysis, isotopic labelling experiments such as those of Suzuki et al. (2001) return data, which are consistent with a single decay constant for Rubisco turnover. Furthermore, the Irving and Robinson (2006) model predicts Rubisco synthesis rates, which correlate very strongly with transcript abundances, and is completely consistent with Lattanzi et al.'s (2005) steady-state labelling experiment to understand N remobilization in ryegrass plants.

Leaf N content tends to be higher in plants grown at higher irradiances except in plants grown at very low N availabilities, although the allocation of N to Rubisco did not change with light intensity in rice leaves (Makino et al., 1997a). Chlorophyll content, however, appeared to vary with light intensity, with plants grown at low irradiances exhibiting increased chlorophyll levels. A similar lack of large differences in N allocation as a result of changes in the fluence rate has also been observed in both *Chenopodium* and *Alocasia* (Hikosaka and Terashima, 1996). Another,

more recent study showed that neither light intensity nor temperature affected the proportion of N invested in Rubisco; however, under conditions likely to promote oxidative stress (low temperature and high light intensity), *Plantago* exhibited decreased chlorophyll levels (Hikosaka, 2005b). Light seems to have little effect on the partitioning of N within the leaf, yet is certainly necessary for the greening process, as leaves grown in the dark do not green up. Similarly grass leaves produce the majority of their Rubisco as they emerge from the previous leaf sheath, with even fairly large leaves containing little Rubisco before emergence.

Whole-leaf Rubisco content increases rapidly during leaf expansion, followed by a peak around full leaf expansion and a subsequent decline in the leaf Rubisco concentration (Mae et al., 1983). The loss of Rubisco post-full leaf expansion follows an approximately exponential decrease, with the maximum rate of Rubisco loss occurring a few days after full leaf expansion. As the chloroplast numbers decrease only slightly during this period, it implies that the majority of Rubisco degradation and N loss during this period are attributable to the degradation of proteins within the chloroplast and export of proteins from the chloroplast by RCBs (Ishida et al., 2008). This was proposed to be a more economically viable method of facilitating the remobilization of proteins from senescent leaves to new tissues than whole-chloroplast senescence. Rubisco concentrations are often in excess for immediate photosynthetic requirements (Warren and Adams, 2004), a phenomenon which may have been exacerbated in the last century by increases in atmospheric CO_2 partial pressures, and photosynthesis is limited by light interception and BuBP regeneration. However, this raises the question of why Rubisco, and presumably other proteins, is so strongly expressed to levels higher than those required for photosynthesis in expanding leaves. Some authors have hypothesized that "excess" Rubisco may represent a store of N for plants (Lawlor, 2002); however, one could argue that such a store would be suboptimal in terms of plant growth, since that N could be better used to increase the leaf area and thus energy interception by the plant. To minimize the expenditure of energy in protein synthesis, while still maintaining high photosynthetic rates, surely the optimal strategy would be for plants to express Rubisco at lower levels, but then maintain Rubisco contents in the leaves for a longer period, without the peak followed by rapid decline in Rubisco content around full leaf expansion notable in grass leaves. This would free up some N for growth, perhaps leading to larger leaf areas or increased tillering. However, neither in rice (Mae et al., 1983), wheat (Mae et al., 1989), barley (Friedrich and Huffaker, 1980), *Arabidopsis* (Wada et al., 2009) nor in ryegrass (*Lolium perenne*; LJ Irving and

C. Matthew, unpublished) do we see any sort of plateau, rather we see a sharp peak, followed by approximately exponential decline in Rubisco content. This suggests that Rubisco contents cannot be precisely regulated and that degradation proceeds at some predefined rate. Initial processing by some protease located inside the chloroplast may be responsible for this. Indeed, as mentioned above, isolated chloroplasts, incubated in the dark exhibited degradation of the RBCL (Kokubun et al., 2002). The quantity of degradation products increased in a time-dependent manner, suggesting a limiting concentration of the active agent, and temperature and pH dependence suggest a protease. Other investigations in tobacco suggest that a DNA-binding protease, CND41 (chloroplast nucleoid DNA binding protein), may be the initial Rubisco protease (Kato et al., 2004). In most species, N is remobilized from the lower, older leaves to younger leaves, yielding a canopy gradient in N content. However, tobacco CND41 antisense mutants exhibit an unusual N distribution, with the soluble protein levels approximately equal between young expanding leaves and older leaves. Furthermore, while the control plants showed a gradient in their leaf Rubisco contents, from youngest to oldest, the antisense plants did not exhibit these patterns, suggesting a suppression of even the initial stage of Rubisco degradation. This work highlights the importance of N remobilization from old leaves to younger leaves, with young CND41 antisense leaves having significantly lower protein levels than the control plants (Kato et al., 2004). The rate of proteolysis in isolated chloroplasts was estimated by Kokubun et al. (2002) at 4% of the starting concentration of the large subunit per day, very similar to Irving and Robinson's (2006) estimates of 3.5% per day modelled from isotopic labelling studies. It is notable that in Kokubun et al. (2002), the Rubisco degradation products accumulated, suggesting that chloroplasts are unable to further degrade Rubisco, and presumably other proteins, by themselves. Given these facts, the Irving and Robinson (2006) model presented an alternate hypothesis to explain excess Rubisco; given that grasses seem unable to closely regulate their rate of Rubisco degradation, and that photosynthetic rates correlate with Rubisco concentrations, over-investment in Rubisco may be a strategy by the plant to maintain maximal photosynthesis for a longer period of the leaf lifespan, and thus maximize photosynthetic gains per unit of C and N invested in a leaf.

Rubisco is generally understood to be protected from degradation when it is in its catalytically active form, or when bound to CA-1-P (Khan et al., 1999). Kato et al. (2004) showed that while urea-treated inactive Rubisco could be readily degraded by the CND41 protease, activated Rubisco was less susceptible to degradation. The urea treatment noted would cause the

denaturation of inactive Rubisco, which presumably allowed its degradation. Interestingly, however, the optimal pH for the activity of CND41 is in the acidic range, while chloroplast stromal pH is typically slightly alkali. Pre-treatment of Rubisco in acidic media increased its degradation rate by CND41, and it is unclear quite how CND41 acts in the chloroplast, although changes in chloroplast redox state through time may play a part. Similarly, Martinez et al. (2008) showed that the pH of their SAVs was in the acidic range, around 5.8.

Nutrient limitation has been shown to increase the rate of decline in leaf protein concentration. This is normally posited as an increase in the degradation rate; however, there is little evidence for this position. The net leaf protein concentration is determined by the rates of protein synthesis and degradation, and a change in either of these will affect the leaf protein concentration. Although some factors such as leaf sugar levels appear to have effects on RCB production, it seems likely that a part of these changes in protein concentrations is caused by declines in protein synthesis rates.

Although nutrient limitation increases the net loss of Rubisco and other proteins, leaf lifespan has been shown to be increased for leaves of some species grown at lower N availabilities (Aerts, 1989), although opposite patterns, or a lack of difference, have been shown in other species (Aerts and Decaluwe, 1995). Oikawa et al. (2006) noted that *Xanthium canadense* plants grown at high N availabilities had a significantly longer leaf lifespan than plants grown under low N levels. All other things being equal, reduced leaf lifespan will reduce the amount of light captured by a leaf, and hence its total C gain. Older leaves tend to contain less N as it is remobilized through time, notably the leaf Rubisco content decreases rapidly after full leaf expansion (Mae and Ohira, 1981; Mae et al., 1983), and this correlates with the reductions in photosynthetic rate (Makino et al., 1983). Despite this, Oikawa et al. (2006) clearly showed that the cumulative net C gain of leaves correlates with time, implying that increase in leaf lifespan would lead to more C being fixed by that leaf.

The mechanism by which N availability extends leaf lifespan is unclear; however, two potential general mechanisms can be posited. We know that C sufficiency can promote senescence, and both high and low N leaves should be able to equally accumulate C—if anything, high N leaves should accumulate more C due to their higher levels of photosynthetic proteins. This suggests that either the C/N ratio is important or N acts in some way to shift the balance towards protein synthesis, thus delaying senescence. The quantity of Rubisco synthesized by a plant depends on its environmental conditions, with high N plants producing more

Rubisco than their low N counterparts (Makino et al., 1984b). While the low N leaves in Makino et al.'s (1984b) study appeared to senescence earlier than the high N leaves, the curve shapes between the high, medium and low N leaves were very similar, suggesting that the control of leaf Rubisco content by N operates by regulating synthesis rather than degradation. Removing N from the growth medium of wheat leaves led to significant decreases in both the leaf Rubisco content and the RNA transcript abundance (Crafts-Brandner et al., 1998). While direct C : N ratio sensing is not an obviously bad idea, in practice it seems harder to understand exactly how such a system would function. Early senescence of leaves grown under low N is easier to understand, especially if the proteolytic rates are fairly constant, where the leaves simply run out of protein to degrade earlier than higher N controls. Similarly, some system relying on leaf C status itself would seem relatively easy to imagine.

N and other nutrients are transferred from older plant parts to newer ones during senescence. The proportion of N supplied for new leaf growth from the turnover of proteins in older leaves is generally considered to be between 60% and 100% in rice (Mae, 1986), although perhaps a little lower around 40% in ryegrass (Lattanzi et al., 2005). The remaining nutrients must be supplied by uptake from the roots. A recent review paper (Hikosaka, 2005a) suggests a method by which light intensity may indirectly affect the proportion of nutrients under varying nutrient conditions. At high irradiances with high nutrient availability, growth is rapid and supported by both nutrient uptake and remobilization. At high irradiance, but low nutrient availabilities, growth is rapid, but its nutrient demands are supported solely by remobilization, causing an increase in old leaf senescence rate. At low irradiances and high nutrient availabilities, the plants grow slowly, with the nutrient demand met by uptake, repressing or at least not promoting, old leaf senescence. Finally, at low irradiances and low nutrient availabilities, growth is very slow, with the nutrient demand of the growing tissues being met by remobilization from older leaves and new assimilation being both energetically costly and unnecessary to support growth rates. Some evidence exists that plants growing under sufficient light but at low nutrients have a higher stomatal conductance, which is suggested to be a mechanism by which the plants can increase their nutrient capture (Cramer et al., 2008). It is unclear how much of this effect is purely due to the increased transpiration rate, or and how much can be attributed to increases in photosynthesis fuelling increased membrane nutrient transporters in the root. Irrespectively, simply taking nutrient supply and growth rate into consideration, this could largely account for the behaviour that we see in plants grown under various nutrient regimes.

Shading of fully expanded leaves appeared to retard the rate of Rubisco degradation in fully expanded rice leaves (Hidema et al., 1991), suggesting a role for light in this process. This seems to run contrary to Hikosaka et al.'s (1996) work discussed below, which clearly showed that shading would tend to decrease the protein levels in vine leaves. There seem to be several possibilities for explaining Hidema et al.'s (1991) data: (i) plant response to light and shading is species-specific, with rice simply behaving in a different way to *Ipomoea*; (ii) light stress is an important causal factor for protein degradation in rice and (iii) unshaded leaves gained more C than shaded leaves, causing increased proteolytic rates. Unfortunately, it is not easy to decide between these possibilities although the third seems most likely. Interestingly, levels of other enzymes were also affected by the shading treatments. While NADP-G3PDH followed a similar pattern to Rubisco, with full light leaves exhibiting more rapid loss of protein than their shaded counterparts, cytochrome *f* contents were oppositely affected, with shaded leaves exhibiting a more rapid loss of protein than unshaded leaves. Chlorophyll contents decreased more rapidly in unshaded plants. No difference in the proportion of N allocated to Rubisco was noted in the treatments, suggesting that the differences in leaf protein levels accurately reflected the total fluxes of N in the leaf.

Deficiency of nutrients other than N can also alter the amount of Rubisco and presumably other proteins in plants. For example, S starvation has been shown to lead to a large decrease in the amount of Rubisco, and a smaller decrease in the total protein levels, in rice leaves. An increase in light stress was also noted in S-deficient rice (Lunde et al., 2008). In *Lemna minor* fronds, S deficiency, but not the deficiency of other nutrients, including N, led to a large decrease in the Rubisco concentration (Ferreira and Teixeira, 1992).

V. IMPLICATIONS OF PROTEIN TURNOVER IN WHOLE PLANTS

Protein turnover is understood to have an important role in recycling N and other nutrients within the plant in such a manner as to increase photosynthesis (Grindlay, 1997) and therein reproductive success. Some fraction of leaf N will be lost during senescence and remobilization, and that represents lost photosynthetic capacity; however, remobilization of N to a new leaf can increase whole-plant photosynthesis, especially in dense canopies of homogenous plants, such as crops. Under N-limiting conditions, a leaf will senesce when the quantity of C it can

fix drops below the amount of C that could be fixed by that N in a new leaf, taking the C cost of N lost during senescence into account. Recent evidence has shown precisely this phenomenon in *Xanthium canadense* plants grown under low N conditions (Oikawa et al., 2006). This can lead to leaves senescing even when they are still able to maintain a positive photosynthetic rate. Under N-sufficient or N-excess conditions, however, such a strong requirement for N remobilization to maximize photosynthetic gain is unnecessary, and indeed under N replete conditions plants seem to retain greater concentrations of N in senescent leaves than are necessary for the amount of photosynthesis performed by those leaves (Oikawa et al., 2008).

In relation to C gain per unit N, plant canopies seem to pursue an optimal strategy, remobilizing N from lower, shaded leaves, to upper leaves where the light intensity is higher. There are two possible explanations for this, which I shall term *deliberate* optimization and *inherent* optimization. *Deliberate* optimization would be defined as the plant having some kind of mechanism by which it can, based on its internal conditions (N content, etc.), adjust and regulate leaf lifespan and N remobilization in such a way as to specifically and deliberately increase whole-plant photosynthesis. The mechanisms for this are obscure, but it is not implausible that such mechanisms could exist—we know from earlier sections that considerable plasticity exists in the regulation of protein synthesis and degradation. *Inherent* optimization suggests that optimization of canopy photosynthesis is simply an emergent property of the system, and that plants have limited capacity to adjust their leaf N contents to the ambient conditions after the termination of leaf expansion. Protein synthesis occurs during leaf expansion, while degradation occurs throughout the leaves' lifespan. Leaves deeper in the canopy (lower on the stem) are older than leaves nearer to the light, at least in grasses, and contain less N simply as a result of their age. This sets up a gradient in N concentration down through the canopy, approximately matching that for light. However, plants which have protein turnover rates allowing the older leaves to senescence and new leaves to grow in the pattern closely resembling the light gradient, thereby maximizing photosynthesis, will tend to outcompete plants with sub-optimal leaf properties and come to dominate the stand. Thus, creating the illusion of deliberate optimization of leaf N, as only the plants with optimal protein turnover rates for the environment will be present. Of course, as noted throughout the text, plant leaves certainly have some capacity to alter their rates of protein synthesis and degradation in respect to their environmental conditions, but these can be explained largely as the changes of cellular behaviour based on their local conditions, rather than the

global changes to the whole plant. Indeed, in the vine *Ipomoea tricolor* grown horizontally to avoid shading, a developmental gradient in leaf N was noted in plants grown at low and intermediate N availabilities, with the youngest leaves having the highest N concentration, while the oldest leaves had the lowest concentrations. If the plants goal is the optimization of C fixation, then all other things being equal, N should have been distributed evenly among the leaves (Presland and McNaughton, 1984). On the other hand, shading of older leaves was able to exacerbate the N gradient evident at low N concentrations, while causing a gradient in older leaves grown at higher N availabilities. Stronger shading tended to produce stronger gradients, and inverse shading—where young leaves were shaded while old leaves were unshaded—produced an inverse N distribution, with old leaves behaving like young leaves and *vice versa*. Indeed, this clearly shows that both N and light can have strong effects on the N distribution between leaves, perhaps even more so than the developmental gradients. It is unclear whether other species share such plasticity with vines.

A recent paper using a barley quantitative trait loci (QTL) mapping population presented results indicating a positive relationship between the efficiency of N remobilization, as characterized by the difference in N concentration between leaf maturity and leaf death, in the flag leaf and grain yield (Mickelson et al., 2003). Plants with large flag leaves were more efficient at N translocation, with leaf weight positively correlated with grain yield. The physiological basis for differences in N remobilization noted by Mickelson et al. (2003) are not currently understood, nor are the reasons for genotypic specificity. However, another paper from the same group looking at the effects of a QTL involved in determining seed N concentration found broadly similar results, with leaf N remobilization from leaves correlated with grain N levels (Jukanti and Fischer, 2008). Recent work has demonstrated a more causal link between leaf N turnover and plant growth (E Khaembah, PhD Thesis, Massey University, NZ). Leaf Rubisco content was quantified through time and modelled using the Irving and Robinson (2006) model for 135 ryegrass (*Lolium perenne*) genotypes in a QTL mapping population. The maximum Rubisco content correlated negatively with leaf length, tiller weight and plant dry weight. This suggests that plants which invest less N into Rubisco tend to have larger leaves, bigger tillers and a higher overall plant mass. Speculatively, plants investing less N into each leaf may be able to support more leaves, increasing light-use efficiency and total C fixed on a whole-plant basis in an N-limiting environment with low levels of competition. Where competition for light is higher, faster turnover rates and higher maximum Rubisco contents may be favoured. Negative relationships were

also noted between the maximum Rubisco content and the standard deviation of the log-normal model and the peak time. Plants which had a high-peak Rubisco content reached that peak content early in the leaf's lifespan, then exhibited a rapid decrease in the Rubisco content, while those which had lower, later peaks also had a more modest subsequent loss of Rubisco.

Plant parasitism may have strong influences on the host-plant leaf condition and on the nutritional status of the plant (Irving and Cameron, 2009). Parasites can be split broadly into two groups: phloem-feeding parasites and xylem-feeding parasites. Phloem-feeding parasites tend to be wholly dependent on their hosts for C (holoparasites), while xylem-feeding parasites tend to absorb water, hormones and nutrients, yet photosynthesize independently (hemiparasites). This was hypothesized to have differential effects on host tissues, and holoparasitized host plants often exhibit retarded leaf senescence (Hibberd et al., 1999), presumably because carbohydrates do not accumulate in their leaves. Hemiparasites may be expected to promote leaf senescence, since they intercept N in the xylem before it reaches the host leaves. Indeed, parasitized *Phleum* plants had significantly less chlorophyll and a slight decrease in leaf Rubisco contents (Cameron et al., 2008).

VI. CONCLUSIONS

N remobilization is hugely important for plant development and growth. In this chapter, we have attempted to give a broad overview of the processes of protein synthesis and degradation, and the factors which seem important in their determination. In grasses, protein synthesis mainly takes place during leaf expansion, with various factors important in regulating leaf protein concentration, specifically N or other nutrients and some hormones such as cytokinins responsible for an increase in leaf protein levels, while C and ABA downregulate synthesis. Light appears to be instrumental in the synthesis of many leaf proteins. The integrated and co-ordinated behaviour of the chloroplast and the nuclear genomes is important, and the mechanisms by which it is affected remain incompletely understood, although progress is being made. Protein degradation is a complex process, with multiple degradation pathways posited for many proteins. The loss of stromal proteins seems to be at least partly a result of vesicular export from the chloroplast, although the mechanism of this export remains deeply contentious. Similarly, the mechanism of degradation of the D1 protein remains contentious, as we have shown here. Reactive oxygen species seem important in D1 degradation and indeed can also cause the

degradation of Rubisco. While it is unclear how much of a role that reactive oxygen species have in natural senescence, it is clear that they certainly have the potential to bleach the cells in carotenoid-less plants, whether this is a result of a mutant gene (*immutans Arabidopsis*) or whether it is a result of the application of carotenoid-suppressing chemicals. The relative amounts of protein synthesized and degraded throughout the lifespan of the leaves determine the protein contents of the leaves. It is well documented that plants exhibit an apparently optimized N distribution in their canopy, although the mechanisms by which these are affected remain unknown. Studies using parasitized plants suggest that the behaviours noted in the plants can be explained by shifts in the C and N levels within the plants. Finally, several recent studies have shown evidence of a strong link between the rates of N remobilization within the plant and its productivity, a conclusion which reinforces the need for continued work in understanding the processes controlling plant N levels.

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